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Laser-assisted cell removing (LACR) technology contributes to the purification process of the undifferentiated cell fraction during pluripotent stem cell culture

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ABSTRACT

Purification of undifferentiated cells by removing differentiated parts is an essential step in pluripotent stem cell culture. This process has been traditionally performed manually using a fine glass capillary or plastic tip under a microscope, or by culturing in a selective medium supplemented with anti-differentiation inhibitors. However, there are several inevitable problems associated with these methods, such as contamination or biological side-effects. Here, we developed a laser-assisted cell removing (LACR) technology that enables precise, fast, and contact-less cell removal. Using LACR combined with computational image recognition/identification-discriminating technology, we achieved automatic cell purification (A-LACR). Practicability of A-LACR was evaluated by two demonstrations: selective removal of trophoblast stem (TS) cells from human iPS and TS cell co-cultures, and purification of undifferentiated iPS cells by targeting differentiated cells that spontaneously developed. Our results suggested that LACR technology is a novel approach for stem cell processing in regenerative medicine.

1. Introduction

Pluripotent stem cells such as embryonic stem (ES) cells and induced pluripotent stem (iPS) cells are considered ideal sources for cell transplantation therapy [1,2]. The major advantage of pluripotent stem cells is their unlimited proliferative capacity maintaining multiple differentiation potentials. On the other hand, *in vitro* culture to expand pluripotent stem cells still possess difficulty; especially, spontaneous differentiation has been an inevitable problem. In the past decade, several methods to improve pluripotent stem cell culture have been suggested. For example, supplementation with chemical inhibitors that block molecular cascades that trigger differentiation is widely used for maintaining pluripotency with uniformity in mouse stem cells [3–5]. Unfortunately, their effects are not reproducible in human pluripotent stem cells, since these signaling cascades are often vital for self-renewal of human ES and iPS cells. Recently, chemically defined mediums optimized for pluripotent stem cell culture have been developed, but several problems exist. Adaptability to the medium is different based on the cell line [6], and the media can affect the metabolism of stem cells [7] or induce cellular stress [8]. In addition, these methods are often highly expensive compared with those using classic medium. Considering the above concerns, traditional manual removal of differentiated cells using glass-capillary or plastic-tips under a microscope remains an effective strategy for maintaining the quality of human pluripotent stem cells [9,10]. However, such techniques require proficiency to discriminate differentiated cells as well as precision in handling. Furthermore, the risk of bi-directional contamination (*i.e.*, between researcher

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and cell cultures) is also a major concern in the manual manipulation approach.

Here, we developed a laser-assisted cell removing (LACR) technology with near infrared radiation (NIR) for purifying undifferentiated cells in human pluripotent cell culture. In addition, we also developed computational identification-discrimination technologies to achieve automated cell maintenance.

2. Materials and methods

2.1. Setting of NIR laser on an inverted microscope and irradiation of human iPS cells

LED with 1460 nm emission was used to generate the NIR laser. The NIR laser generator and lens for focusing and targeting were originally developed. The lens for NIR emission was set on to an inverted microscope (CKX53, Olympus, Tokyo, Japan). In the present experiment, total scanning line of the NIR laser was fixed at 40 mm/mm^2 (1 mm horizontal scanning was repeated 40 times vertically per 1 mm^2) and the scanning speed was set at 15 mm/s. The human iPS cell line SeV-K1 originally established with Sendai virus [11] was maintained in a Nutristem medium (Biological industries., Cromwell, CT, USA). The human iPS cells were irradiated with a 1460 nm NIR laser at 10–400 mW for 1.3 msec (0.013 mJ–0.52 mJ). After NIR irradiation, the cells were stained with Calcein-AM (TAKARA Bio, Shiga, Japan) and propidium iodide (TAKARA Bio).

2.2. Thermometry of the medium surrounding the NIR focal point

Evaluation of heat accumulation was performed for 220 mW, 1.3 msec (0.29 mJ)/40 μm-spot diameter laser conditions, which was consistent with the laser settings used for subsequent cell manipulations. Thermometry was performed using a Thermo GEAR G120EX thermography (Nippon Avionics Co., Ltd., Tokyo, Japan) with a Thermocouple wire sensor (TT-T-36, OMEGA Engineering, Tokyo, Japan), by which data updating cycle was 0.1 s. During the measurement, the thermo-sensor was placed on the bottom of the culture dish in 4 mL of Nutristem medium.

2.3. Electron microscopy

Morphological studies on the LACR-treated samples were performed using scanning electron microscopy (SEM; SU3500, HITACHI., Tokyo, Japan). The fixed specimens were freeze-dried with VFD-20 (Shinkuu device Co., Ltd, Ibaraki, Japan) and mounted with an ion sputter (E-1030, HITACHI).

2.4. Automated cell recognition, discrimination, and LACR for trophectodermal stem (TS) cells in co-culture with human iPS cells

Image capturing was performed using a 4 Megapixel CMOS CCD camera (GS3-U3-41C6C-C, FLIR Integrated Imaging Solutions Inc., Richmond, BC, Canada). After identification of colony boundaries, binarization was performed using Savola's thresholding method, wherein the threshold is determined using the mean and standard deviation of the local pixel intensities [12]. The position and size of the nuclei were computationally estimated on the basis of nucleolar regions with low pixel value (black) and karyoplasm with high pixel values (white) enclosed by the computationally defined boundaries. Cytoplasmic area of the iPS cells was estimated on the basis of the distance between adjacent identified nucleoli. Cell density was estimated from the number of nucleoli per unit area. An algorithm to differentiate between undifferentiated iPS cells and TS cells/differentiated cells was designed on the basis of integrated results of the scores for the pseudo-nuclear-cytoplasmic ratio, cell density, brightness, and colony morphology. Targeting of the cells was achieved by moving the culture dish using an originally developed electric stage (supplementary Figure 1).

A mouse TS cell line was established from the B6D2F1 mouse (CLEA Japan, Tokyo, Japan) embryo, as previously reported [13]. The TS cells were transfected with a pPB-EGFP plasmid and used to co-culture with the human iPS cells. The established TS cells and the human iPS cells were co-cultured on Matrigel™ (BD Biosciences, San Jose, CA, USA)-coated petri dishes in a Nutristem medium. At 48 and 72 h after cell seeding, the culture dish was treated with A-LACR. The LACR condition was 220 mW, 1.3 msec (0.29 mJ) for a 40 μm-spot diameter, which corresponded to the temperature measurement condition. After irradiation, cells were stained with propidium iodide and analyzed by multicolor flow cytometry assays with BD LSR Fortessa (BD Biosciences).

2.5. Removing spontaneously differentiated cells from iPS cell cultures by A-LACR

We then evaluated the applicability of the A-LACR for purification of undifferentiated cells during normal pluripotent cell cultures. The human iPS cells maintained in the hPS medium on MEF were harvested on Matrigel™-coated petri dishes and cultured in a Nutristem medium for 72 h. After A-LACR, improvements in cell culture were evaluated by qPCR assays for pluripotent cell markers *NANOG*, *POU5F1*, *SOX2,* and *KLF4*.

2.6. Quantitative RT-PCR (qRT-PCR) analysis

cDNA was prepared using a TRI Reagent® (Molecular Research Center, Inc., OH, USA) and PrimeScript® RT Master Mix Kit (TAKARA Bio Inc.). Quantitative real-time PCR with total cDNA was performed using Perfect real-time SYBR green II (TAKARA Bio Inc.). The following primers were used for the qPCR:

NANOG sense 5′-TAGTGAAAACTCCCGACTCTG-3′, antisense 5′-AGCTGGGTCTGGGAGAATAC-3′

POU5F1 sense 5′-AGTCACTGCTTGATCGTTTG-3′, antisense 5′-AAGAACATGTGTAAGCAGCG-3′

SOX2 sense 5′-CGTTCATCGACGAGGCTAA-3′, antisense 5′-CGGTATTTATAATCCGGGTGC-3′

KLF4 sense 5′-GCAGAAGACACTGCGTCAAG-3′, antisense 5′-AGTCGCTTCATGTGGGAGA-3′

GAPDH sense 5′-AAGTATGACAACAGCCTCAAG-3′, antisense 5′-TCCACGATACCAAAGTTGTC-3′.

All values are means ± SD of 3 experiments. Statistical significances were evaluated by Student's t-test with JMP software version 10.0.0 (SAS Institute, Cary, NC, USA).

3. Results

3.1. Irradiation of 1460 nm NIR laser induced cell death in human iPS cells

To observe the effect of NIR laser irradiation on cultured iPS cells, we set an LED illuminant source that can generate a 1460 nm wavelength to a microscope and targeted the quadrilateral area of human iPS cell culture. At 5 min post treatment, the irradiated areas were stained with PI indicating that NIR irradiation could damage cultured cells. After 24 h of the NIR irradiation, targeted cells were detached (Fig. 1A). We then determined the optical condition for efficient cell destruction. The efficiency of cell death depended on both the power of exposure and the spot diameter (Fig. 1B). When the cells were exposed to 100 m W (W) NIR or lower, there were many surviving cells in the target region, as measured by PI

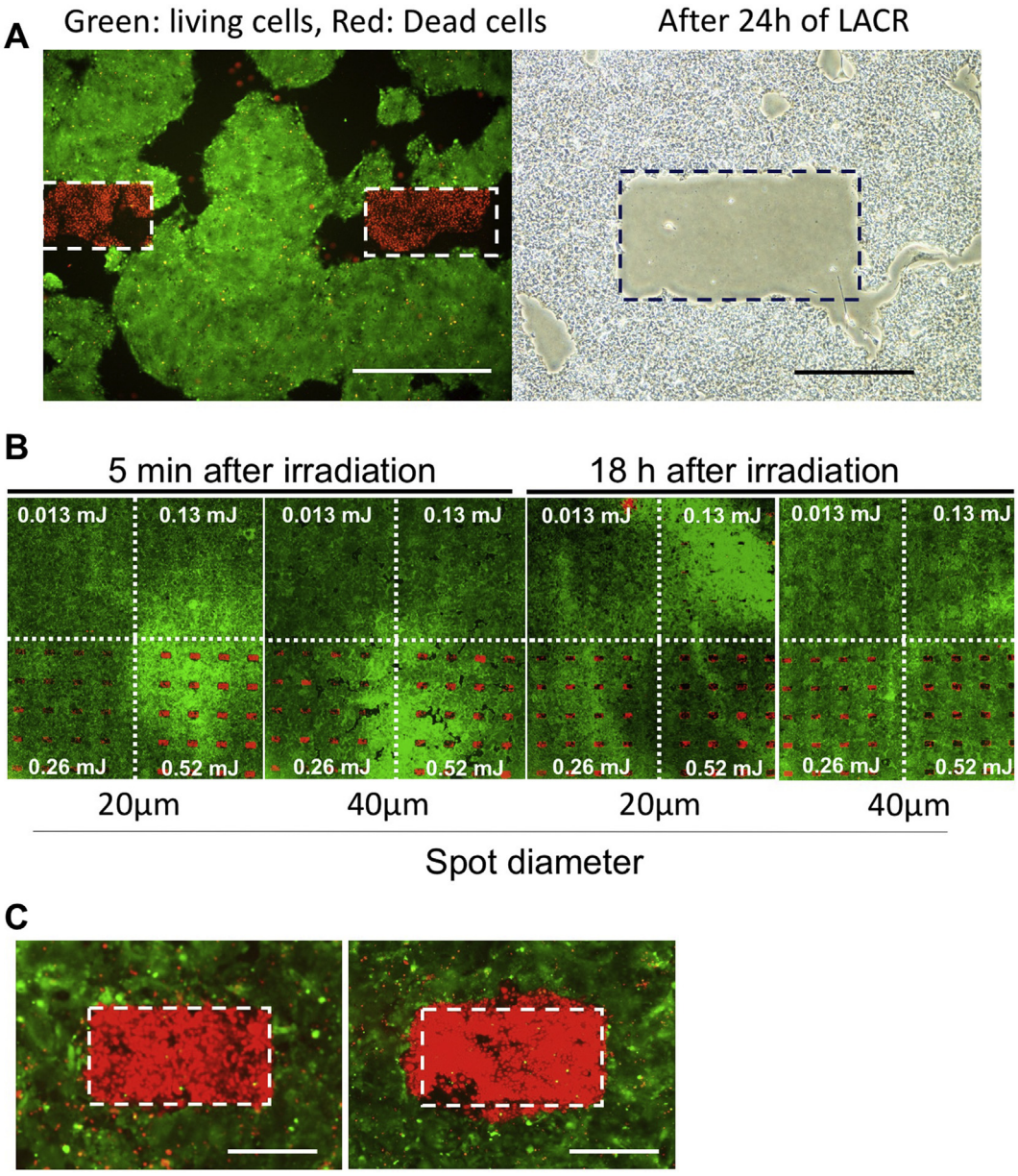

Fig. 1. Cell destruction by NIR-laser irradiation and detection of an optimal irradiating condition. A. Cell viability test with Calcein-AM (Surviving cells) and PI (dead cells) for the 1,460 nm NIR laser-treated cells. Scale bar = 1 mm. Targeted cells were detached from the culture dish by washing after 24 h of the irradiation. Scale bar = 0.5 mm. B. Optimization of laser irradiation condition. C. Highly magnified images of the Calcein/PI-stained specimens. Left panel is a representative image of the cells irradiated with 200 mW for 1.3 msec (0.26 mJ) and right panel shows the area irradiated with 400 mW for 1.3 msec (0.52 mJ). Scale bar = 0.5 mm.

staining. Contrary, when the cells were exposed to 400 mW for 1.3 msec (0.52 m J [J]), peripheral cells that were out of the actual targeted area were also destroyed (Fig. 1C). After further optimization of the irradiating condition, we determined that 220 mW for 1.3 msec (0.29 mJ)/40 μm-spot diameter were the optical conditions for cell removal in iPS cell culture.

3.2. Temperature increasing in the medium surrounding the targeted area

To confirm the heating effect of NIR irradiation on the medium surrounding the cells targeted, we measured the medium temperature during NIR irradiation. An increase in temperature was observed soon after laser exposure, reaching around 36 °C from 25 °C when the focal point was mostly closed to the sensor (Fig. 2A). This indicates that a temperature increase can occur in the medium surrounding the targeted cells during NIR irradiation, and it can reach up to a 11 °C increase in the medium surrounding the targeted area with LACR (Fig. 2B).

3.3. LACR treatment manually improved the purity of undifferentiated cells in human iPS cell culture

We next examined whether LACR treatment was available to remove differentiated cells from human iPS cell culture without affecting the viability of undifferentiated cells. PI staining and SEM observation demonstrated that the targeted differentiated cells were destructed and died soon after the LACR treatment (Fig. 2C and D), but the undifferentiated cells neighboring the targeted cells remained undamaged (Fig. 2E). The remained iPS cells maintained

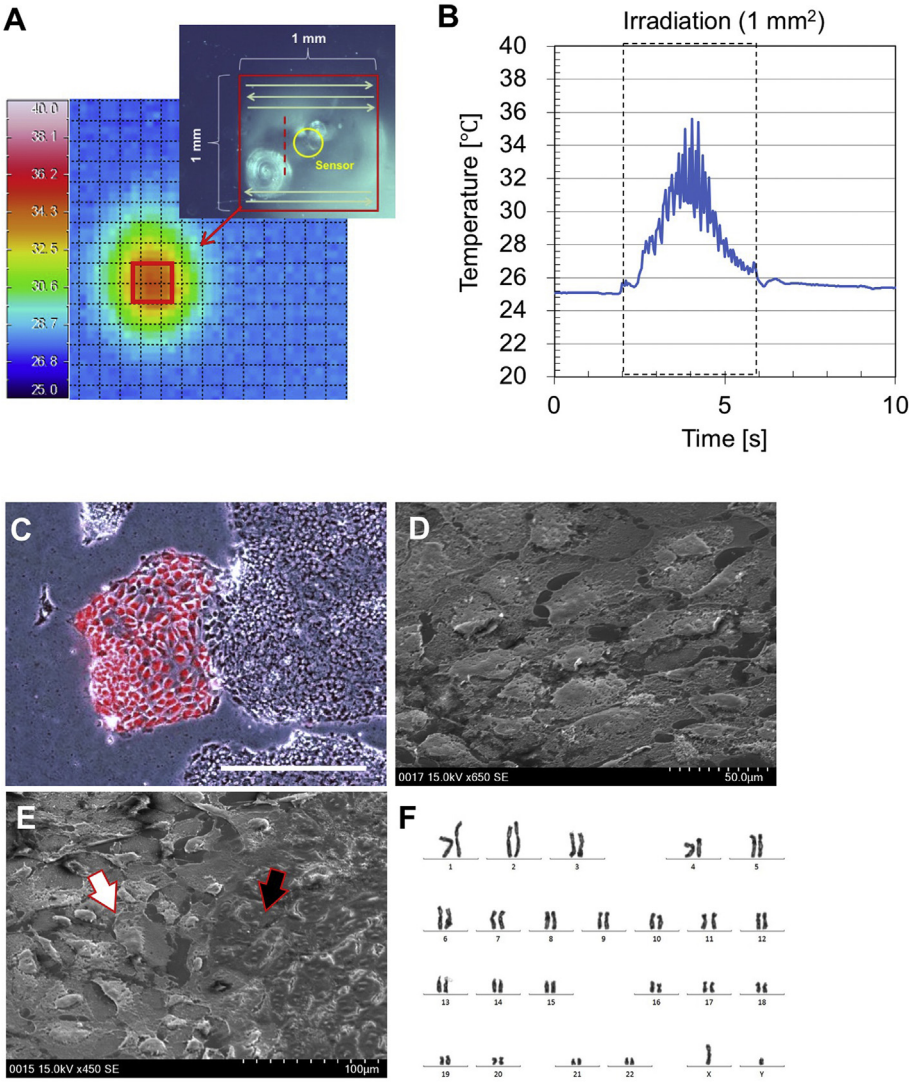

Fig. 2. Temperature measurement of the irradiated area. A. Setting of the thermo-sensor in the culture dish. This test was performed in the 1 mm × 1 mm square. The NIR laser was sequentially irradiated in the direction of the arrow at 25 μm interval. B. Thermometry in the irradiated area. C. Microscopic observation of the undifferentiated cell colony neighboring the targeted cells stained with PI. Scale bar = 500μm. D. SEM image of the irradiated cells. E. SEM image of the boundary region. Black arrow shows non-irradiated surviving cells and white arrow shows irradiated cells. F. Karyotype of the remained iPS cells.

normal karyotype when analyzed at 72 h after treatment (Fig. 2F). These results show that LACR is an effective tool to remove unnecessary cell parts from cell culture.

3.4. Automated-LACR (A-LACR) recognized TS cells and removed them from human iPS cell culture automatically

Then, we developed a system to automatically recognize differentiated cells and remove them from iPS cell culture based on the above notion (Fig. 3A). In the present settings, it takes approximately 20 min for image capturing, analysis, and designing irradiation area, and laser irradiation per 60 mm^2 dish containing targeting object at 5% in 80% confluent iPS cells. When verifying its precision using TS cells that were co-cultured with iPS cells, the A-LACR system successfully distinguished TS cells: the percent of GFP-positive cells showing TS cells was decreased from approximately 26% to 8.1% (N = 3, Fig. 3C and D).

Then, we investigated whether A-LACR was useful for removing spontaneously developed differentiated cells from the iPS cell cultures. When human iPS cells were passaged as small pieces of colonies, a small subset of the cells often differentiated spontaneously and disturbed the uniformity of the pluripotent stem cell cultures. In this experiment, we performed A-LACR treatment 3 days after passaging of human iPS cells. The computational recognition system successfully discriminated between undifferentiated cells and differentiated cells (Fig. 4A), and the differentiated cells were selectively destroyed by subsequent LACR. Quantitative RT-PCR demonstrated that the gene expression levels of pluripotent cell markers *NANOG*, *POU5F1*, *KLF4*, and *SOX2* significantly increased in the A-LACR-treated iPS cells (Fig. 4B).

4. Discussion

The effects of cell transplantation therapy are thought to mainly depend on the purity and quality of the transplants, which are critical factors that influence clinical results [14,15]. Especially, pluripotent stem cells are vulnerable to deterioration of the culture environment. Therefore, maintaining a high-purity of undifferentiated cells is highly important for ES and iPS cell culture. In this study, we aimed to establish novel methods for removing

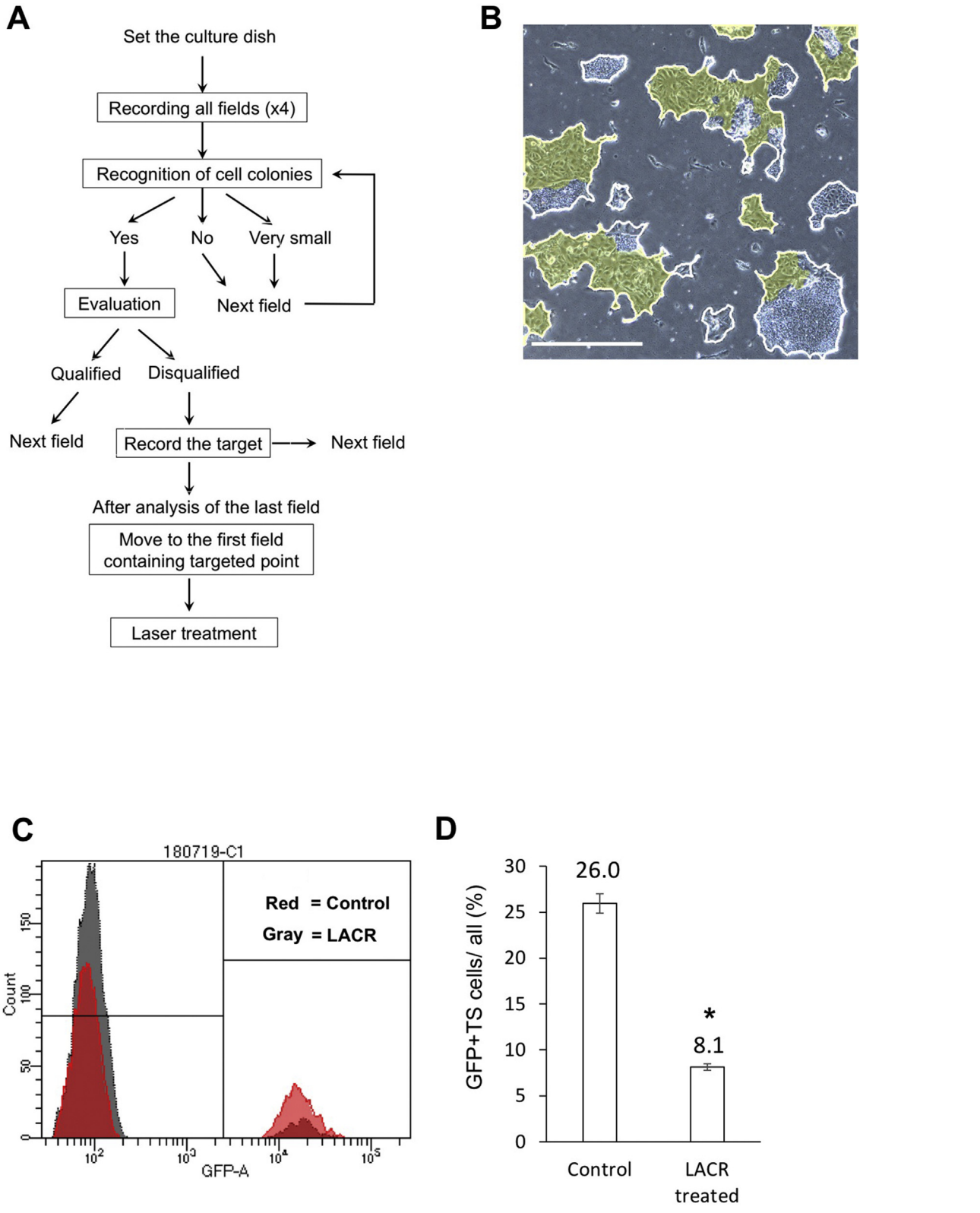

Fig. 3. Automatic-LACR (A-LACR) development and evaluation in the TS/iPS cell co-cultures. A. Outline of the automatic cell recognition-evaluation and -irradiation program developed in the present study. B. Automatically identified area for LACR treatment (yellow). Scale bar = 1mm. C. FACS analysis after A-LACR treatment of the TS/iPS cell co-cultures. D. Rate of GFP-positive TS cells in control and A-LACR treated cultures. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

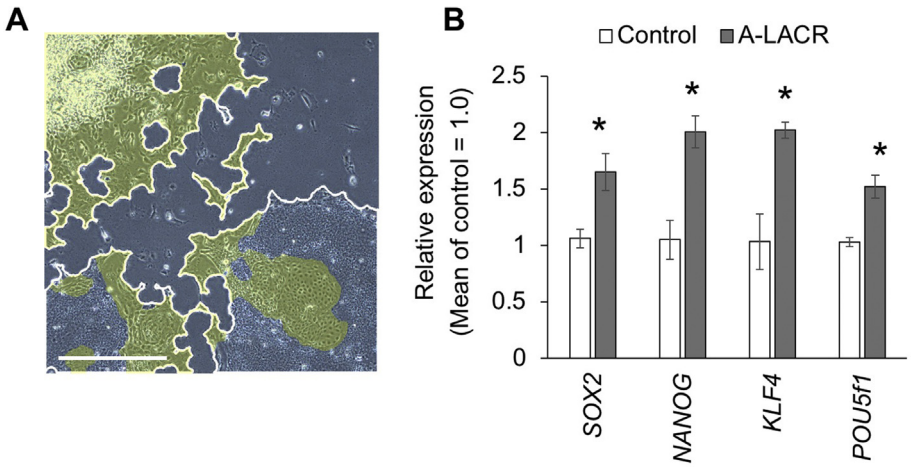

Fig. 4. A-LACR treatment for removing differentiated cells in iPS cell culture. A. Automated identification of the target area. Differentiated cells were successfully marked as target (yellow, Scale bar = 1 mm, estimated value). Scale bar = 1 cm. B. qPCR for pluripotent cell markers for evaluation of effectiveness of A-LACR to improve iPS cell culture. Bars show the mean value of three independent experiments. Asterisks represent significant differences ($P < 0.05$) between the control. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

unnecessary cells using NIR, and to evaluate the practicability in human iPS cell culture.

Cell manipulation with lasers has already been conceptualized as Laser-Enabled Analysis and Processing (LEAP) technology, wherein cells are assessed using conventional fluorescent and/or bright field image and processed the cells with 532-nm green laser [16]. This technique suggested the possibility of high-throughput cell manipulation; however, certain limitations and potential concerns remained. For example, optimizing the laser conditions for cell removing was essential since lasers at ≤532 nm could result in mutagenesis [17,18]. Furthermore, although availability of laser in the human iPS cell passaging has been represented [19,20], practicability in cell purification, its purity and effect on the processed regions remain to be cleared. To develop new methods to improve the laser-ablation mediated cell processing, we used a laser in the near-infrared spectrum (1460 nm) and developed an automatic cell recognition-evaluation method with non-labeling phase-contrast imaging.

In the beginning, we determined the optimal laser conditions, which were defined by NIR-laser power and irradiation time, and concluded that 0.26 mJ for a 40 μm-spot diameter is enough to destroy the target cells. By this method, it was possible to destruct the targeted area at almost the single cell level. On the other hand, it was concerned that sequenced irradiation may result in the heat accumulation in the medium. To clear this, we monitored the temperature of the medium near the targeted area. The temperature at a 20-μm distance from the center of the focal point possibly increased by 11 °C from the baseline, means that if the irradiation was performed under normal culture conditions (37 °C), the medium temperature could likely exceed 42 °C. Therefore, the medium temperature needs to be kept under 25 °C before NIR laser irradiation. Maintaining the manipulation temperature under 25 °C is not technically difficult. Moreover, previous evidence indicates that human pluripotent stem cells possess high-tolerance to extended durations of at least 48 h of exposure to 4 °C and 25 °C [21]. Therefore, we concluded that the NIR laser was applicable for cell removal in human pluripotent stem cell cultures. For practical application of the LACR into routine stem cell maintenance, we developed an automated cell removing (A-LACR) technology, in which differentiated cells are computationally identified and irradiated by NIR sequentially. We constructed a computational algorithm to discriminate between the undifferentiated and differentiated state of human iPS cells and ES cells. The software we developed here successfully discriminated and removed both TS cells, which were used as a model of differentiated cells, and the differentiated cells naturally developed from iPS cells. Applicability to human ES cell cultures was also tested and its accuracy was determined with the rBC2LCN-FITC probe, which binds only to undifferentiated ES/iPS cells (supplemental Figure 2). Furthermore, subsequent analysis revealed that the expression levels of pluripotent marker genes increased in the A-LACR-treated iPS cells. These results show that the A-LACR technology enables automatic purification of human pluripotent stem cells. In the present system, we adopted analysis with phase contrast images. Using this system, numerous pictures can be analyzed quickly and inexpensively without cell invasiveness, since it is not needed to construct complicated structures of multiple optical paths and control systems. Furthermore, our algorithm is based on simple morphological features such as size of the cytoplasm and nuclei, cell density, and colony morphology. This characteristic allows for ease of modification and optimization in accordance with cell lines or cell types. Although it is still under development, our technology may be utilized for other stem cell cultures such as mesenchymal stem cells, in which contamination of other cell types frequently occurs and could lead to loss of stemness [22].

On the other hand, present method includes two important limitations. First, accuracy of image analysis tended to decrease at the edge region of the culture dish, because intensity of phase contrast is degraded around the dish wall. Although we designed our evaluation program to strictly limit assessments in the regions, in which the phase contrasting did not work well, complete elimination of unnecessary cells was impossible, as was evident in the study using TS/iPS cell co-cultures. Overcoming this limitation requires the procuring of other imaging parameters and/or techniques, or removing all cells existing in the unavailable region. The second concern is un-known side effects of NIR. However, NIR is already used in some clinical cases, particularly for *in vitro* fertilization (IVF). For IVF, NIR is used for cutting or thinning the zona pellucida, which is a glycoprotein shell surrounding human oocytes that protects them from physiological stress, to assist with hatching and implantation of cryopreserved embryos or those with poor prognosis [23,24]. In the assisted hatching, approximately 0.15–1 mJ per 8 μm spot diameter of a 1450 nm NIR laser was used for human embryos [25–27]. Accordingly, numerous healthy

babies are born from embryos treated with NIR [28]. This indicates that NIR itself may not induce significant genetic effects. As for the thermal damage caused by heat absorption in the medium surrounding the cells, some evidence suggests that embryos exposed to lasers do not exhibit retardation in cell growth [29,30]. Clinically, there is further evidence showing no increase in congenital malformations after the NIR laser procedure [31].

Although long-term data on stem cell culture using lasers is not yet available, LACR technology has clear advantages: LACR enables non-contact cell manipulation without generating and exchanging hazardous particles, thus maintaining a high level of cleanness. This also results in operator safety when processing patient samples, which are likely to contain pathogenic materials. Accessibility to a closed culture system is also an important feature of LACR. LACR can penetrate the plastic wall if it is color-less, and it is possible to easily remove unintentionally emerging cells in the flasks.

Recently, various novel technologies to support clinical cell processing have been developed. Automatic cell culture systems utilizing robotic technologies are one of the most attractive approaches [32,33]. In automated cell culture, quality control is a key aspect for improvement and for clinical application. LACR may prove to be a valid option for assisting the above-mentioned technologies.

Contributions

T.T, K.M. K.S, and H.S. were responsible for conceptualization. T.T and H.S. designed the studies. K.M., K.S., Y.M., and Y.M. constructed the programs and instruments. T.T, T.T, T.M., and H.S. performed biological experiments. T.T and K.F. provided data analysis and interpretation. T.T and S.H. wrote the manuscript. All authors contributed to and approved the final manuscript.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.bbrc.2018.08.101.

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